Adaptive mutation: General mutagenesis is not a programmed response to stress but results from rare coamplification of *dinB* with *lac*

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In a particular genetic system, selection stimulates reversion of a *lac* mutation and causes genome-wide mutagenesis (adaptive mutation). Selection allows rare plated cells with a duplication of the leaky *lac* allele to initiate clones within which further *lac* amplification improves growth rate. Growth and amplification add mutational targets to each clone and thereby increase the likelihood of reversion. We suggest that general mutagenesis occurs only in clones whose *lac* amplification includes the nearby $dinB^+$ gene (for error-prone DNA polymerase IV). Thus mutagenesis is not a programmed response to stress but a side effect of amplification in a few clones; it is not central to the effect of selection on reversion.

When a particular *lac* mutant of *Escherichia coli* is plated on selective medium, revertant colonies accumulate over several days (1, 2). Two models assume that mutations arise in the nongrowing population (3). Directed mutation proposes that stress preferentially induces beneficial (i.e., Lac^+) mutations (1, 2). The hypermutable state proposes that stress induces general (genome-wide, undirected) mutagenesis in a subset of cells (≈0.1%), and this mutagenesis produces the Lac^+ revertants (4–6).

An alternative model, amplification mutagenesis, proposes that reversion occurs in cells growing under selection and requires no change in mutability. On selective medium, rare preexisting cells with a *lac* duplication initiate slow-growing clones within which the growth rate increases progressively as amplification increases the copy number of the partially functional mutant *lac* allele (7, 8). The probability of reversion within each clone increases with the number of target *lac* copies. After reversion, selection holds the revertant lac^+ allele and favors cells that stabilize this allele by loss of mutant copies. This model is a specific form of a more general hypothesis proposed by Lenski *et al.* (9).

Genomewide mutagenesis (an ≈100-fold increase in rate) is experienced by some revertants. This mutagenesis depends on the error-prone DNA polymerase, DinB (10–12), which may be induced when the SOS regulon is activated by DNA fragments released during segregation of the amplified *lac* region (8). Three problems complicate understanding how selection might cause general mutagenesis:

(*i*) Induction of SOS does not mutagenize strains with a single wild-type $dinB^+$ gene (13–15).

(*ii*) Only 10% of Lac^+ revertants arising under selection experience general mutagenesis (16, 17).

(*iii*) Selection causes mutagenesis only when *lac* is near the *dinB* gene (16, 18–21).

Evidence is presented that general mutagenesis occurs only in those developing clones whose amplified *lac* region includes the nearby $dinB^+$ gene. Thus general mutagenesis is not a programmed response to stress in stationary phase but rather a side effect in a subset of developing clones growing under strong selection.

Materials and Methods

Supporting Information. More detailed descriptions of methods and complete genotypes of all strains are published as supporting information on the PNAS web site, www.pnas.org.

Strains. Except where noted, strains are derivatives of the *Salmonella enterica* (serovar Typhimurium) strain LT2. Throughout, plasmid F'_{128} refers to a derivative carrying a triply mutant *lac* allele that includes a deletion fusing the *lacI* and *lacZ* genes (Ω), a mutation improving the *lacI* promoter (I^Q), and a +1 frameshift mutation (*lacI33*) (2). Transposon *Mu*dCF (22) includes this mutant *lac* operon. The *lexA*(null) and *lexA*(Ind^-) mutations and the *sulA* and prophage deletions required for viability of a *lexA*(null) mutant were described previously (20, 23).

Media and Chemicals. Rich medium was nutrient broth (NB; Difco), and minimal medium was either E-glucose or NCE-lactose (24). Other growth media and antibiotic concentrations were described (20, 22, 23). 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (Diagnostic Chemicals, Oxford, CT) was used at 25 μg/ml in minimal medium and, for colony sectoring tests, at 40 μg/ml in NB.

Construction of Mutations by Linear Transformation. Most recipients carried plasmid pKD46, which encodes recombination functions of phage λ (*red*, *gam*, and *exo*) expressed from an arabinose-inducible promoter (25, 26). The chromosomal *dinB* gene of *S. enterica* was replaced by the Rif^R gene (*arr-2*) expressed by the *cat* gene promoter of pACYC184. Mutations were made on F'_{128} in the region that includes the genes *dinB mhpC lac prp* (27). The *dinB* gene was replaced with a Kan^R determinant (F'_{128} *dinB62*::Kan). Two duplications were constructed, each with a Cam^R determinant at its join point. One duplication (24 kb) includes both *dinB* and *lac* and was constructed as shown in Fig. 1*A*. The other (21 kb) includes *lac* and *prp* but not *dinB* and was made in an analogous way. A Rif^R determinant was used as a selective marker to insert the *E. coli* $dinB^+$ gene and its regulatory region into the *lacA* gene of the MudCF element (Fig. 1*B*). For details, see supporting information.

Lac Reversion Tests. Strains were pregrown in NCE glycerol with necessary supplements (28), washed in NCE medium, and plated (2×10^8 cells) with a 10-fold excess of Lac^- scavenger cells (*S. enterica* LT2) on NCE lactose medium containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and needed amino acids. Scavengers consume carbon sources other than lactose and prevent growth of the reversion tester strain. Plates were incubated 5–8 days at 37°C, and revertants (Lac^+) were counted daily. Plotted numbers are the mean number (with standard deviation) of revertants per plate from 10 plates.

Abbreviation: NB, nutrient broth.

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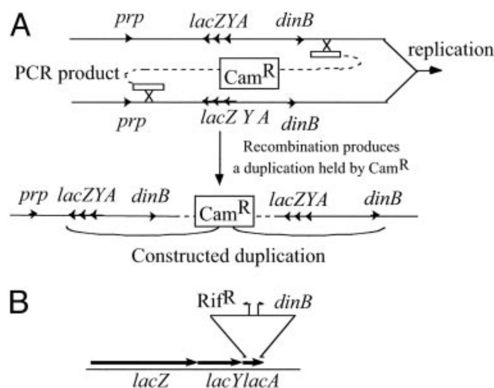

Fig. 1. (*A*) Construction of a *lac* duplication by linear transformation. The introduced PCR fragment includes a Cam^R cassette flanked by sequences identical to separated regions of the recipient F'_{128} plasmid. This fragment can recombine to generate a duplication. (*B*) Structure of a *lacA*::(din*B*, Rif) insertion.

Assaying Mutagenesis. Mutagenesis was scored as the fraction of Lac$^+$ revertants that carried an unselected mutation in any of 100 genes, determined as described (20).

Identifying Unstable *lac*$^+$ Cells in Revertant Colonies. Each revertant colony was resuspended in NCE, diluted, and plated for single colonies on NB, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (40 μg/ml). Within 3 d, unstable Lac$^+$ cells form colonies that are blue with many white (Lac$^-$) sectors; strains with this phenotype carry a tandem array of *lac* copies (7, 8, 29–31).

Transfer of F'_{128} from Unstable Lac$^+$ Cells into *recA* Cells. Revertant colonies appearing on day 5 were suspended, diluted, and plated on NB–5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; unstable Lac$^+$ colonies (sectored) were used directly to inoculate cultures for mutation rate measurement (below). To determine the *lac* copy number, each revertant plasmid was transferred to recipient strain DA7700 (*recA1*, *proB*, *leu*, *srl*::Tn*10*dCam). Transconjugants (Pro$^+$) were selected, and Lac$^+$ clones were identified by their blue color. For Southern hybridizations, DNA was prepared from one blue colony from each mating. Amplified tandem arrays are stable without selection in the *recA* background (7). Reported copy numbers are a minimal estimate of amplification, because some segregation could occur before stabilization.

Southern Hybridization Analysis of Gene Copy Number. DNA was prepared by using a Qiagen (Chatsworth, CA) DNA preparation kit, cut with *Hin*cII, separated on a 1% agarose gel, vacuum-blotted to Hybond N filters, and hybridized with ^{32}P-labeled probes. Probes (300 bp) were prepared by PCR amplification and radioactively labeled with ^{32}P-CTP by using a random priming kit (ReadyPrime, Amersham Pharmacia Biotech). Hybridization was performed and quantified as described (7). The chromosomal *S. enterica dinB* gene does not contribute to estimates of the *dinB* copy number.

Determining Mutation Rates. Ten tubes of NCE medium (1 ml) supplemented with glycerol (0.2%) and LB (1%) were inoculated with 10^2 cells and incubated overnight. Cells were washed in NCE and plated (0.1 ml) on NB rifampicin plates (80 μg/ml) and (0.2 ml) on NCE lactose (0.2%) plates with 10^9 scavenger cells (LT2). Mutants (Rif^R) were counted after 24 h and Lac$^+$ revertants, after 48 h.

Mutation rates in *recA*$^+$ strains with a *lac* amplification were measured by selecting resistance to the pyridine analogue 6-amino nicotinamide (6ANm). Ten to 20 tubes of NCE lactose, leucine medium (1 ml) with or without mitomycin C (0.4 μg/ml), were inoculated (10^4 cells), grown overnight, and plated (0.1 ml) on E-glucose medium containing 6ANm (50 μg/ml). Resistant colonies (observed after 48 h) are primarily due to *pncA* or *pncB* null mutations (32). The viable cell number was determined on NB. Mutation rates were calculated from the median number of mutants per viable cell (33).

Results

Definitions. The term "adaptive mutation" refers here to the process by which selection increases the number of Lac$^+$ revertants. This definition makes no assumptions regarding the role of growth or the contribution of general mutagenesis to reversion. Two aspects of this process are discussed here: reversion and mutagenesis. In this article, "reversion" means mutational correction of a particular *lac* allele to *lac*$^+$ under selective conditions defined for this system (2). By "mutagenesis," we mean a genome-wide undirected increase in mutation rate occurring during the process of reversion under selection, detected as unselected mutations carried by Lac$^+$ revertants.

Selection Is Not Mutagenic When *lac* Is on Conjugative Plasmids Other Than F'_{128}. In the original system, the *lac* mutation is on plasmid F'_{128}, which includes a *dinB*$^+$ gene. We moved the mutant *lac* region to two conjugative plasmids that lack a *dinB* gene: F'_{152} *nadA* and the resident *Salmonella* plasmid pSLT. Reversion of *lac* on these plasmids is indistinguishable from that of *lac* on plasmid F'_{128} (22). Unlike F'_{128}, these plasmids allow normal *lac* reversion to occur without mutagenesis (parentheses in Fig. 2 *A* and *B*). Thus mutagenesis is unnecessary for selection-stimulated *lac* reversion. Although these plasmids have no *dinB* gene, they are carried in strains with a chromosomal *dinB*$^+$ allele. Thus a chromosomal *dinB*$^+$ allele is not sufficient for mutagenesis under selection in this system.

Selection Is Mutagenic if *dinB*$^+$ Is Near *lac* on a Conjugative Plasmid. The entire *dinB*$^+$ gene of *E. coli* was inserted (with its regulatory region) into the nonessential *lacA* gene of plasmid F'_{152} *nadA* (*lac*), described above, restoring mutagenesis and slightly increasing the yield of Lac$^+$ revertants (Fig. 2*A*, numbers in parentheses). The small effect of restored mutagenesis on reversion is consistent with the conclusion that mutagenesis is at best a minor factor in adaptive mutation.

Similarly, removal of the *dinB*$^+$ gene from the F'_{128} plasmid in *S. enterica* eliminated mutagenesis but reduced reversion only ≈4-fold (Fig. 3*A*). The same reduction is seen in *S. enterica* and *E. coli* when SOS induction is prevented and in *E. coli* when both plasmid and chromosomal *dinB* alleles are inactive (11, 12, 20, 34). This 4-fold effect of mutagenesis on reversion is tiny compared with the 10^4-fold effect of growth and amplification (see below).

The Chromosomal *dinB*$^+$ Gene Is Irrelevant to the Reversion Rate. Fig. 3*B* describes three pairs of strains carrying F'_{128} and differing only by a chromosomal *dinB* null mutation. Strains in the top pair carry a *lexA*(null) mutation, which causes constitutive SOS expression; this pair shows a slightly enhanced reversion compared with those in the second pair, which are *lexA*$^+$. The third pair lacks a *dinB* allele on the F′ plasmid and shows 4-fold reduced reversion even though its *lexA*(null) mutation causes constitutive SOS expression of any remaining *dinB* gene. In all three pairs, the chromosomal *dinB* allele had no effect on reversion. The critical *dinB*$^+$ allele appears to be near *lac* on the conjugative plasmid. The conflict between these results (in *S. enterica*) and those in *E. coli* (10, 11) is addressed below.

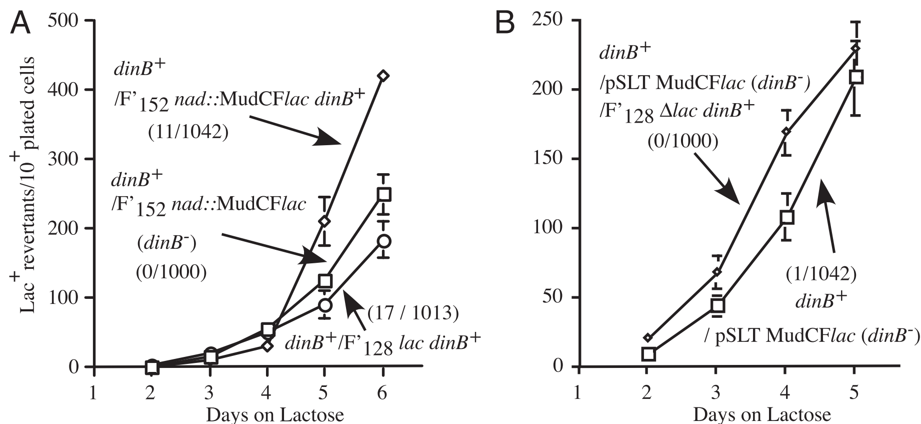

Fig. 2. Plasmids other than F'_{128} support *lac* reversion without mutagenesis. Parentheses enclose the fraction of Lac$^+$ revertants with an unselected mutation. (*A*) The F'_{152} *nadA*::MudCF plasmid carries the original *lac* allele with (TT20850) or without an added *dinB*$^+$ gene (TT24446). A control strain carries the original F'_{128} *lac dinB*$^+$ plasmid (TT18302). (*B*) The strain in the lower curve (TT23009) carries pSLT MudCF*lac*, which has the *lac* allele but not *dinB*. The strain in the upper curve (TT24581) has *lac* on pSLT MudCF*lac* and *dinB*$^+$ on F'_{128} *lac*Δ *dinB*$^+$.

Having *dinB*$^+$ on a Conjugative Plasmid Is Not Sufficient for Chromosome Mutagenesis. DinB-dependent mutagenesis is not seen in *E. coli* unless the *dinB*$^+$ gene is highly overexpressed (13, 14). Similarly in *S. enterica*, constitutive expression of a chromosomal *dinB*$^+$ gene is not mutagenic during nonselective growth (23), suggesting that the mutagenesis seen in the *lac* system might occur simply because the strain used has a few extra *dinB*$^+$ copies, one copy in the chromosome and one on each of the several copies of F'_{128} *lac* (2). SOS induction of these extra copies might allow DinB mutagenesis with or without selection.

During nonselective growth of these strains, neither the *lac* reversion rate nor the rate of mutation to rifampicin resistance (Rif^R) was increased by constitutive SOS expression, even in cells with several copies of *dinB*$^+$ (Table 1). Cells of *S. enterica* carrying *lac* in the chromosome were grown under nonselective conditions and tested for their rates of *lac* reversion and mutation to Rif^R. SOS induction was provided by a *lexA*(null) mutation. Revertants were counted after 2 days on selective medium and therefore reflect the mutation rate during nonselective pregrowth (Table 1). During long-term exposure to selection, these strains yielded very few *lac* revertants, even with the extra copies of *dinB*$^+$ on an F′ *lac*Δ *dinB*$^+$ plasmid (data not shown); this may reflect difficulty in amplifying the chromosomal *lac* region (22). Thus having *dinB*$^+$ on a conjugative plasmid is necessary (see above) but not sufficient for mutagenesis, even with SOS induction. Both *lac* and *dinB*$^+$ must be on a conjugative plasmid, and selection must be imposed.

Selection is mutagenic when *lac* and *dinB*$^+$ are on the same conjugative plasmid. This mutagenesis is eliminated when a *lexA*(Ind$^-$) mutation prevents SOS induction (12, 20). A *lexA*$^+$/ F'_{128} *lac dinB*$^+$ strain showed both reversion (Fig. 3*B*) and mutagenesis (19 of 1,013 Lac$^+$ revertants carried an unselected mutation). An isogenic *lexA*(null) mutant, constitutive for SOS (Fig. 3*B*), showed slightly more revertants and more unselected mutants (40 per 1,000 Lac$^+$ revertants). Thus mutagenesis is associated with reversion when three conditions are met: (*i*) *lac* and *dinB* are on a conjugative plasmid, (*ii*) selection is imposed, and (*iii*) SOS is induced.

Mutagenesis (but Not Reversion) Requires That *dinB*$^+$ Be Located *cis* to *lac* on the Same Plasmid. Although mutagenesis was seen when *dinB*$^+$ and *lac* were both on the same plasmid, it is not clear that this *cis* positioning is essential. To test this requirement, the *lac* and *dinB*$^+$ genes were placed on different conjugative plasmids. The mutant *lac* allele was inserted in plasmid pSLT, where selection stimulates reversion without causing mutagenesis (Fig. 2*B*). The *dinB*$^+$ allele was on plasmid F'_{128}, where it stimulates mutagenesis under selection when *cis* to *lac* (Fig. 2*A*). For this

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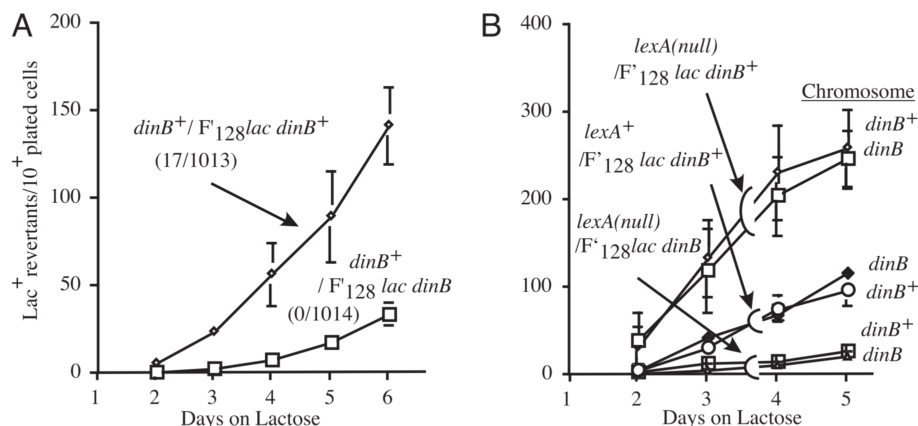

Fig. 3. Effect of *dinB* deletions on Lac$^+$ reversion and mutagenesis. Data are the mean and standard deviation of five independent experiments. All strains carry a *sulA* mutation and lack three prophages, Gifsy-1, Gifsy-2, and Fels-2, to allow viability of a *lexA*(null) mutant (23). (*A*) Strains used were TT18302 (F'_{128} *lac dinB*$^+$) and TT23664(F'_{128} *lac dinB*::Kan). (*B*) The strains were TT24461 [*lexA*(null) *dinB*$^+$/F′*lac*$_{128}$ *dinB*$^+$], TT24463 [*lexA(null) dinB*/F′*lac*$_{128}$ *dinB*$^+$], TT24462 (*lexA*$^+$, *dinB*/F'_{128} *lac dinB*$^+$), TT24460 (*lexA*$^+$ *dinB*$^+$ F'_{128} *lac dinB*$^+$), TT24456 [*lexA*(null) *dinB*$^+$/F'_{128} *lac dinB*], and TT24458 [*lexA*(null) *dinB*/F'_{128} *lac dinB*].

Table 1. Effect of *dinB* copy number on mutability

Strain	Genotype*		*dinB* copy no.	Unselected mutation rate ($\times 10^{-8}$)	
	Chromosome (all $dinB^+$)	$F'_{128}\Delta$lac plasmid		Lac^+ revertants†	Rif^R mutants
TT24597	$lexA^+$	$dinB^+$	3	0.5	0.7
TT24598	*lexA41*::Cam	$dinB^+$	3	0.4	0.5
TT24599	$lexA^+$	*dinB*62::Kan	1	0.5	0.3
TT24600	*lexA41*::Cam	*dinB*62::Kan	1	0.4	0.4

*Chromosome: *proB1657*::Tn*10 sulA46*::Spc *nhaC1*::MudCF.
†Reverting *lac* allele is within chromosomal *nhaC*1 insert.

experiment, the *lac* gene was deleted from F'_{128}, so the only *lac* allele under selection is that on pSLT (*trans* to $dinB^+$). Mutagenesis is not seen when $dinB^+$ is *trans* to *lac*, either in the chromosome (above) or on a different conjugative plasmid (Fig. 2*B*).

A Model for Mutagenesis Under Selection. The above results suggested that mutagenesis might require coamplification of *dinB* with *lac* during growth under selection. Selection enhances reversion primarily by favoring growth of cells with an amplification of the mutant *lac* gene (8), which is only 16.6 kb away from *dinB* on F'_{128} (27). We propose that in some clones, the amplified *lac* region happens to include $dinB^+$. Multiple $dinB^+$ copies, when induced by SOS, may produce sufficient mismatches to overwhelm the methyl-directed mismatch repair system and cause mutagenesis. Several predictions of this model were tested.

Prediction 1: The *dinB* Gene Is Included in Some Spontaneous *lac* Amplifications. Cells exhibiting an unstable Lac^+ phenotype were identified in late-arising revertant colonies and were used to inoculate cultures for determination of mutation rate (below) and *lac* amplification. Amplification was measured after stabilization of the tandem array by conjugative transfer of the F'_{128} *lac* plasmid to a *recA* mutant recipient. Twenty-five independent *lac* amplifications were tested by Southern hybridization for their content of *lac*, *dinB*, and a chromosomal control gene *cheY*. Five of these 25 included *dinB*; eight representative amplification strains are shown in Table 2. In the five amplifications that included *dinB*, the ratio of *lac* to *dinB* varied from 1.7 to 3.7; one might have expected a 1:1 ratio. The underrepresentation of *dinB* may reflect either its loss from some repeated units or the formation of secondary *lac* duplications lacking *dinB* during growth under selection for amplification of *lac*.

Prediction 2: SOS-Induced General Mutation Rates Are Higher in Strains with a $dinB^+$-*lac* Amplification. Mutation rates (to $6AN^R$) were measured in three amplification strains, two with $dinB^+$ and one without *dinB* in the repeated unit (Table 3). These $recA^+$ strains were grown selectively with or without mitomycin C (to induce SOS, as occurs during selective growth on lactose with competing scavenger cells). As inferred above, mutagenesis required SOS induction, and the increase was higher in strains with a $dinB^+$ amplification (30- to 60-fold) than in strains with an unamplified $dinB^+$ gene (6-fold).

Table 2. Some amplified *lac* arrays include *dinB*

Strain*	Genotype of $F'_{128}lac$	Relative copy number		
		cheY	*dinB*	*lacZ*
DA5199	No amplification	1	1	1
DA8044	Unstable Lac^+	1	1	102
DA8045	Unstable Lac^+	1	1	66
DA8092	Unstable Lac^+	1	22	58
DA8095	Unstable Lac^+	1	4	11
DA8114	Unstable Lac^+	1	14	37
DA8115	Unstable Lac^+	1	10	17
DA8156	Unstable Lac^+	1	1	52
DA8157	Unstable Lac^+	1	17	63

*Chromosome: *proB1657*::Tn*10, leuD21, srl-203*::Tn*10*dCam, *recA1*.

Table 3. Amplification of *dinB* increases mutagenesis

Strain	Genotype*,† amplified on F'_{128}	Relative copy number‡			Mutation rate $6ANm^R$ ($\times 10^{-5}$)		Fold increase
		cheY	dinB	lacZ	−MMC§	+MMC	
DA8120	*lac*	1	1	52	0.12	0.67	6
DA8074	*lac* and *dinB*	1	22	58	0.19	11.00	58
DA8121	*lac* and *dinB*	1	17	63	0.16	5.45	34

*Chromosome: *proB1657*::Tn*10 leuD21;* these strains are $recA^+$ derivatives of strains DA8156, DA8092 and DA8157 in Table 2.
†Plasmid F'_{128} carries *lacIq lacI33*(fs) lacIZ (ω fusion).
‡Copy number determined in *recA* strains (Table 2).
§Mitomycin c.

Prediction 3: A Constructed *lac* Duplication Without $dinB^+$ Stimulates Reversion but Not Mutagenesis. A duplication of the *lac-prp* region of plasmid F'_{128}, but not the $dinB^+$ gene, was constructed in the parent strain. This duplication increased revertant yield 100-fold. Dilutions of the duplication strain were plated on selective medium with a constant number (10^9) of scavenger cells. A 100-fold dilution (10^6 cells) gave roughly the same number of Lac^+ revertants seen for undiluted cultures (10^8) cells of the standard strain (Fig. 4).

Revertant colonies appearing in this duplication-bearing parent strain included mostly unstable Lac^+ cells (carrying an unstable *lac* amplification) and a few stable Lac^+ cells that had lost the mutant copies by segregation. This preponderance of unstable types was seen previously whenever mutagenesis was prevented, either by a LexA(Ind^-) mutation (20) or when *lac* was on a plasmid lacking the $dinB^+$ gene (above). Without mutagenesis, reversion is delayed until growth of the amplification clone provides more *lac* copies; this delay prevents haploid Lac^+ segregants from dominating the composition of the revertant colony. In the original strain (with *lac* near $dinB^+$ on F'_{128}),

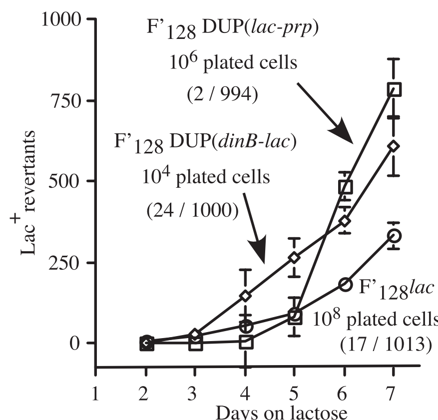

Fig. 4. Preexisting duplications stimulate reversion and (if *dinB* is included) mutagenesis. Note dilutions of the parent populations that carry a preexisting duplication. F'_{128} Dup(*lac-prp*) indicates a strain whose duplication includes *lac* but not *dinB* (TT23666). F'_{128} (*dinB-lac*) indicates a strain whose duplication includes *lac* and $dinB^+$(TT23665). Both duplications have a Cam^R determinant at the join point. The control strain with no *lac* duplication is TT18302 (data replotted from Fig. 2*A*).

mutagenesis occurred, and revertant colonies were dominated by stable Lac$^+$ haploid cells (8), presumably because reversion occurred early.

As predicted, mutagenesis was low when *dinB*$^+$ was not included in the amplified unit. Only two unselected mutants were found among 994 Lac$^+$ revertants tested. Without a given duplication, an average of 16 unselected mutants (14–19) were seen per 1,000 Lac$^+$ revertants.

The 100-fold increase in revertant frequency caused by a *lac* amplification suggested that spontaneous *lac* duplications are carried by ≈1% of the cells in the standard strain before selection. This frequency was confirmed by direct tests (E.S.S., unpublished work) and is ≈10-fold higher than the frequency of typical chromosomal duplications (35), in agreement with an earlier conclusion that plasmid conjugative functions stimulate duplication and amplification, perhaps by generating DNA ends from the transfer origin (22). Thus in the original experiment, 100 revertant colonies develop from an estimated 10^6 plated duplication-bearing cells, suggesting that many plated duplication-bearing cells fail to initiate successful clones and possibly explaining the failure of respreading experiments (3, 31). The formation of 100 revertants from 10^6 plated duplication cells represents a 10^4-fold increase over the *lac* reversion frequency seen during unrestricted growth (10^{-8}). This increase is almost all attributable to amplification and growth, with mutagenesis providing a factor of ≈4.

The constructed duplication used in this experiment carries a Cam^R marker at its join point, making it possible to test the idea that stable Lac$^+$ cells in a revertant colony have lost the *lac* duplication, as predicted by the amplification model. All of 600 unstable Lac$^+$ cells extracted from revertant colonies retained their Cam^R phenotype; all of 600 stable Lac$^+$ cells had lost Cam^R.

Prediction 4: A Constructed Duplication with *lac* and *dinB*$^+$ Stimulates Both Reversion and Mutagenesis. A *lac-dinB* duplication in the F'_{128} plasmid of the parent strain increased the Lac$^+$ revertant frequency 10^4-fold (100-fold more than did a duplication of *lac* alone). These revertants showed a high frequency of associated unselected mutations (Fig. 4). An identical *lac* duplication without a *dinB* gene behaved like the *lac-prp* duplication described above.

A *lac-dinB*$^+$ duplication stimulates revertant frequency for two reasons: (*i*) Every plated cell carries a preexisting *lac* duplication (as was true for duplication of *lac* alone), and (*ii*) every developing clone carries an amplified *dinB* gene and is thus subject to mutagenesis (not true for the simple *lac* duplication). The 100-fold effect of adding *dinB*$^+$ to the amplification suggests that the mutation rate increases ≈100-fold, in agreement with measurements in Table 2 and previous estimates of mutagenesis intensity (16, 20).

In revertants arising from the *dinB*$^+$-*lac* duplication strain, stable Lac$^+$ cells were ≈10-fold more frequent than those arising in the parent whose duplication included *lac* and not *dinB*. This is predicted if the higher mutation rate allows reversion and haploid segregants to arise earlier in the history of the colony and dominate the population after overgrowth.

Recombination Between Plasmid and Chromosome May Explain Some Data Conflicts. Experiments with *S. enterica* (above) suggested that mutagenesis requires *dinB*$^+$ and *lac* to be located *in cis*. McKenzie *et al*. (10, 11) concluded that in *E. coli*, a *dinB* mutation *cis* to *lac* on the F'_{128} plasmid did not reduce reversion rate as long a *dinB*$^+$ allele was present in the chromosome (i.e., DinB seemed to mutagenize even when its gene was *trans* to *lac*). We suggest that, in the *E. coli* experiments, the *dinB* mutation *cis* to *lac* was frequently repaired by recombination between the F'_{128} plasmid and the chromosomal *dinB*$^+$ allele. It is known that DNA ends generated by the plasmid transfer origin stimulate

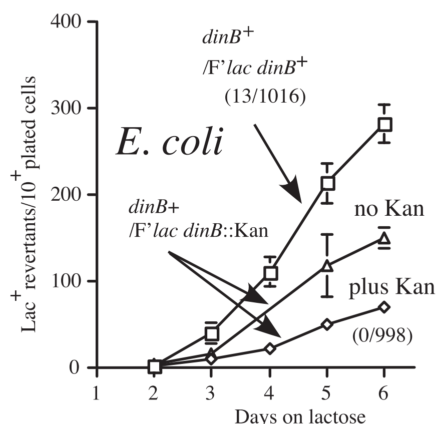

Fig. 5. A *dinB*$^+$ allele *trans* to *lac* cannot support mutagenesis in *E. coli*. The *dinB*$^+$ strain is the original *E. coli* strain (FC40) of Cairns and Foster (2). The isogenic *dinB62*::Kan strain (TT24669) was made by linear transformation.

intense recombination between the plasmid and the identical chromosomal region of *E. coli* (36–38). This recombination is not a problem in *S. enterica* because of substantial sequence differences between the *E. coli* genome fragment carried by F'_{128} and the corresponding region of the *S. enterica* chromosome.

This idea was tested by using an *E. coli* strain that has a *dinB*::Kan swap mutation on F'_{128} and a *dinB*$^+$ allele in its chromosome. In reasonable agreement with McKenzie *et al*. (10, 11), the *dinB*::Kan mutation *cis* to *lac* caused only a 2-fold reduction in *lac* reversion (Fig. 5). However, 25% of the 200 Lac$^+$ revertants tested had lost the plasmid *dinB*::Kan mutation, presumably by recombination with the chromosome. Loss of *dinB*::Kan^R from F'_{128} was not seen when the same experiment was done in *S. enterica* (data not shown).

The *lac* reversion experiment was repeated in *E. coli* with kanamycin added to select retention of the plasmid *dinB*::Kan mutation. When selectively maintained, the plasmid *dinB* mutation caused a 4-fold reduction in revertants in *E. coli*, just as seen for the same *dinB*$^+$/F′ *dinB*::Kan heterozygote in *S. enterica* (compare Figs. 5 and 3*A*). Furthermore, the plasmid *dinB*::Kan mutation eliminated mutagenesis even though a *dinB*$^+$ allele remained in the chromosome (parentheses in Fig. 5). Kanamycin had no effect on revertant frequency in a *dinB*$^+$/*dinB*$^+$ strain that carried a Kan^R determinant elsewhere in the F'_{128} plasmid (data not shown). We conclude that in *E. coli*, as in *S. enterica*, mutagenesis requires a *dinB*$^+$ allele *cis* to *lac* on the F′ plasmid, and the chromosomal *dinB*$^+$ gene makes no contribution.

Discussion

Mutagenesis is experienced by ≈10% of the Lac$^+$ revertants that arise in this experiment (5, 16). This mutagenesis is neither necessary (Fig. 2) nor sufficiently intense (16, 20, 21) to cause the observed *lac* revertants. The evidence provided here suggests that this mutagenesis is not a programmed cell response to stress (6) but an indirect effect of amplification in those clones whose selectively amplified *lac* region happens to include *dinB*$^+$.

Three Prerequisites for Selection-Induced General Mutagenesis. First, the SOS system (and *dinB*) must be induced. This is inferred because mutagenesis is eliminated in *lexA*(Ind$^-$) mutants, which cannot induce the SOS system (12, 20), and by *dinB* mutations (ref. 12; see also Fig. 3*A*). SOS is induced in part by single strands produced by the transfer origin of the F′ plasmid (K.L.B, unpublished data) and may be further stimulated by DNA fragments released when amplified arrays segregate (8). It is well established that single-stranded DNA induces SOS (39).

Second, the *dinB*$^+$ gene must be located *cis* to *lac* on a

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conjugative plasmid. The plasmid location of *lac* stimulates gene duplication and amplification, presumably by means of DNA ends generated at the transfer origin (22, 28, 40). Having $dinB^+$ *cis* to *lac* allows some of the *lac* duplications to include $dinB^+$.

Third, selection must be imposed, which favors growth of cells with an increased *lac* copy number and, in clones whose repeated unit includes $dinB^+$, indirectly increases the $dinB^+$ copy number. Increased DinB levels make sufficient mismatches to saturate the mismatch repair system (MMR) and cause mutagenesis. Competition between DinB and MMR in mutagenesis fits well with previous evidence that DinB-dependent mutagenesis requires producing DinB from a high copy number plasmid (13, 14) and may explain why overexpression of MutL reduces reversion under selection (41–43).

Two Classes of *lac* Revertants. These results suggest two types of revertant colonies: (*i*) Rare mutagenized clones (10–20% of the total) are initiated by cells whose *lac* duplication (and later amplification) includes $dinB^+$; and (*ii*) common unmutagenized clones (80–90% of the total) are initiated by cells whose *lac* duplication (and later amplification) does not include $dinB^+$. Although SOS induction may occur in both revertant types, only those with increased $dinB^+$ copy number suffer general mutagenesis. In clones with a *dinB* amplification, the mutation rate increases several hundred-fold. Averaged over all clones, the mutation rate increases 20- to 50-fold (20, 33).

These two revertant types have been seen before. Rosche and Foster (16) showed that ≈10% of revertant colonies experience a 200-fold increase in mutation rate, and 90% develop with little or no mutagenesis. This agrees with our finding that 20% of *lac* amplifications include $dinB^+$ and increase the mutation rate. We suggest that the unmutagenized revertants were allowed by *lac* target number increase alone. Hastings *et al.* (31) reported two types of Lac^+ revertant colonies, one with only stable Lac^+ cells and a second with only unstable Lac^+ cells. The first type was attributed to stress-induced mutagenesis and the second, to stress-induced amplification. However, too few cells were tested (50 instead of 4,000) to observe that the colony types differ only in the relative abundance of stable and unstable Lac^+ cells, and that both types form by the same process (duplication, amplification, reversion, and segregation).

Understanding the Effect of Selection on the Mutation Spectrum. The amplification mutagenesis model seems inconsistent with data on the nature of the revertant lesions. The lac^+ reversion events (sequence changes) that occur under selection tend to be −1 frameshift mutation in monotonous base runs, a type made by DinB (44), whereas a wider variety of mutation types arises during nonselective growth (45, 46). How can DinB dictate the *lac* mutation spectrum under selection when 80–90% of clones have no *dinB* amplification?

We suggest that during growth under selection, SOS induction of unamplified *dinB* may cause weak mutagenesis, too weak to be detected as associated mutations by our assay method. If clones with a single $dinB^+$ copy on F'_{128} experienced a 5-fold increase in mutation rate, DinB would be responsible for 80% of sequence changes, which would be sufficient to affect the spectrum of *lac* mutations arising under selection. Weak mutagenesis in clones growing without amplified *dinB* and strong mutagenesis in clones with $dinB^+$ amplification would be consistent with the results presented here and with earlier findings of Rosche and Foster (16).

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